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Role of ion channels and subcellular Ca²⁺ signalling in arachidonic acid-induced dilation of pressurised retinal arterioles

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Abstract

Purpose: To investigate the mechanisms responsible for the dilatation of rat retinal arterioles in response to arachidonic acid (AA).

Methods: Changes in the diameter of isolated, pressurized rat retinal arterioles were measured in the presence of AA alone and following pre-incubation with pharmacological agents inhibiting Ca^{2+} sparks and oscillations and K^+ channels. Subcellular Ca^{2+} signals were recorded in arteriolar myocytes using Fluo-4-based confocal imaging. The effects of AA on membrane currents of retinal arteriolar myocytes were studied using whole-cell perforated patch clamp recording.

Results: AA dilated pressurised retinal arterioles under conditions of myogenic tone. Eicosatetraynoic acid (ETYA) exerted a similar effect, but unlike AA, its effects were rapidly reversible. AA-induced dilation was associated with an inhibition of subcellular Ca^{2+} signals. Interventions known to block Ca^{2+} sparks and oscillations in retinal arterioles caused dilatation and inhibited AA-induced vasodilator responses. AA accelerated the rate of inactivation of the A-type K_v current and the voltage dependence of inactivation was shifted to more negative membrane potentials. It also enhanced voltage-activated and spontaneous BK currents, but only at positive membrane potentials. Pharmacological inhibition of A-type K_v and BK currents failed to block AA-induced vasodilator responses. AA suppressed L-type Ca^{2+} currents.

Conclusions: These results suggest that AA induces retinal arteriolar vasodilation by inhibiting subcellular Ca^{2+} signalling activity in retinal arteriolar myocytes, most likely through a mechanism involving the inhibition of L-type Ca^{2+} channel activity. AA actions on K^+ currents are inconsistent with a model in which K^+ channels contribute to the vasodilator effects of AA.

Introduction

Arachidonic acid (AA) is an omega-6 long-chain polyunsaturated fatty acid. It is a major constituent of neuronal tissues of the retina and brain and represents ~10% of total fatty acids in intact vessels of the bovine retina and confluent endothelial cell/pericyte monolayers.¹

In resting cells, AA is stored within the cell membrane, esterified to glycerol phospholipids. The highest concentrations of AA in the retina are found in phosphatidylcholine and phosphatidylethanolamine.² AA can be liberated via receptor- and Ca^{2+} -dependent and/or Ca^{2+} -independent pathways.³⁻⁵ AA can be rapidly freed from the *sn*-2 position of membrane phospholipids by the enzyme phospholipase A₂.⁶ It can also be generated indirectly by other lipases from lipid products containing arachidonate such as diacylglycerol, anandamide, or 2-arachidonylglycerol.⁶ In many vascular beds, AA is known to be released from the endothelium to affect the overlying smooth muscle cells.^{6, 7} In the brain and retina, however, it can also be released from neuronal and glial cells that surround the blood vessels.⁸⁻¹⁰

AA and its metabolites are believed to be involved in the regulation of blood flow under basal conditions as well as during periods of increased metabolic demand. Vasodilator reactions induced by the electrical stimulation of cremaster muscle¹¹ or cortical perivascular nerve fibres,¹² for example, have been shown to be dependent upon AA signalling. In isolated perfused mesenteric arteries, the vascular response to AA is complex, with time-dependent constrictions and dilations being observed.¹³ In addition, when injected intravenously, AA has been shown to lower systemic arterial blood pressure by decreasing both cardiac output and systemic vascular resistance.¹⁴

There is large body of evidence demonstrating that the effects of AA are mediated by modulation of ion channel function and Ca^{2+} signalling mechanisms.¹⁵ In many vascular beds, AA-induced vasomotor effects have been linked to the modulation of K^+ channel activity, including K_{Ca} , K_{ATP} and K_{v} channels.^{13, 16} AA has also been implicated in the regulation of intracellular Ca^{2+} dynamics in various tissues, including cardiac myocytes and vascular smooth muscle cells,¹⁷⁻²⁰ and has been reported to inhibit several different classes of voltage-dependent Ca^{2+} channels.²¹

Presently, the role of AA in the control of vascular tone in the retina is not well understood. Here, we report that AA induces dilation of isolated, myogenically active rat retinal arterioles. Experiments were also conducted to examine the mechanisms responsible for AA-induced vasodilation by investigating the effects of AA on cell membrane ion channels and sub-cellular Ca^{2+} signalling in retinal arteriolar myocytes.

Methods

Animal use conformed to the standards in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the UK Animals (Scientific Procedures) Act, 1986. Male Sprague-Dawley rats (8-12 wk-old) were sacrificed by CO₂ asphyxiation (electrophysiology data) or injection with a lethal dose of sodium pentobarbital (300 mg/kg of body weight, given intraperitoneally; all other data sets).

Arteriole cannulation and pressurisation

First-order retinal arterioles were isolated from dissected retinas and cannulated as described previously.²² Arterioles were inflated and maintained at 70 mmHg for at least 15 min in normal Hanks' superfusate to allow for development of a stable level of myogenic tone. Intraluminal pressure was regulated by changing the height of a fluid reservoir connected to the inflow cannula and monitored using a pressure transducer. Preparations were leak-tested by observing an air bubble introduced into the tubing connecting the cannula to the transducer. If, at fixed high pressure, the bubble was moving this suggested that the preparation was leaky and the experiment was terminated.

Arteriolar diameter measurements

Changes in arteriolar diameter were recorded using videomicroscopy and analyzed as described previously.²² Briefly, a section of the arteriole, at least 40 µm away from the tip of the cannula was viewed under a 40x, NA 0.6 objective and imaged using a MCN-B013-U camera (Mightex, Pleasanton, US). The average internal diameter for a specified region of interest was measured

using Image J (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD). The output was calibrated in micrometers using a graticule image. In the present study, it was not possible to use either the maximum diameter of the vessels at 70 mmHg in Ca^{2+} free solution or the initial diameter following the pressure step to 70 mmHg as a measure of the maximum passive diameter of the vessels for data normalization. Specifically, in some arterioles the dilation in Ca^{2+} free solution did not reach the initial diameter following the pressure step to 70 mmHg (strongly suggesting that they had not reached their maximum passive diameter), whereas in other vessels the dilation went beyond that initial value (preventing us from using the diameter following the initial pressure step for normalisation). Data has therefore been expressed as a percentage change in diameter from steady-state baseline values in the text and in absolute units in Tables 1 and 3.

Sub-cellular Ca^{2+} imaging

Smooth muscle Ca^{2+} signals in pressurized retinal arterioles pre-incubated with Fluo-4AM (10 μM for 2 h) were imaged using a confocal scanning laser microscope (Bio-Rad, MR-A1, Richmond, USA).²² The Fluo-4 was excited by an argon laser beam (488 nm) and emitted light was filtered through a 530-560 nm bandpass filter and detected using a photomultiplier tube (PMT). Ca^{2+} sparks and oscillations were analysed with custom-made software and their spatiotemporal characteristics measured as described previously.²² Changes in arteriolar diameter could not be simultaneously imaged with the Fluo-4 emitted fluorescence. Therefore, we collected brightfield and confocal recordings sequentially. Two different regions of the same vessel were imaged under control conditions and 5 min after AA treatment.

Electrophysiological recordings

Whole-cell membrane currents were recorded from individual smooth muscle cells still embedded within their parental arterioles using the perforated-patch clamp technique.²³ Supplementary Table S1 provides details of protocols used to isolate individual current components. Pipette resistances were 1-2 MΩ. Membrane currents were recorded using an Axopatch-1D (Axon Instruments) amplifier, low pass filtered at 0.5 kHz and sampled at 2 kHz by National Instruments PC1200 interface using WinWCP (v 3.3.3, J.Dempster, University of Strathclyde, UK) software. The same software was also used for data analysis. Leak currents were subtracted off-line from the active currents using the standard leak subtraction protocol embedded within the WinWCP software. Series resistance (15-30 MΩ) was routinely compensated by >70%. Currents were normalised to cell capacitance and expressed as current densities (pA/pF). Cell membrane capacitance was determined from the time constant of a capacitance transient elicited by a hyperpolarizing step from -60 mV to -80 mV with a sampling frequency of 20 kHz.

The voltage dependence of K_v channel activation was calculated by converting peak currents to a conductance (G) using the following equation: $G = I / (V_m - E_k)$, where: I is the current amplitude, V_m is the command potential and E_k is the equilibrium potential for K^+ . Based on the Nernst equation, E_k was estimated to be -80 mV under recording conditions ($[K^+]_o = 6$ mM and $[K^+]_i = 138$ mM). Values were then normalized to the maximum conductance (G/G_{max}). The voltage dependence of K_v channel inactivation was investigated by holding the retinal arteriolar myocytes at different membrane potentials during a 3 sec conditioning pre-pulse and then applying a common test pulse (+60 mV; 1 sec). The peak current following each test pulse

was expressed relative to the maximum current recorded following a conditioning pre-pulse at -100 mV (I/I_{\max}). The resulting G/G_{\max} and I/I_{\max} values were fitted with a Boltzmann equation.²⁴

Drugs and solutions

The composition of the solutions used was as follows (in mM): (1) Hanks' solution - 140, NaCl; 6, KCl; 5, D-glucose; 2, CaCl_2 ; 1.3, MgCl_2 ; 10, HEPES; pH set to 7.4 with NaOH; (2) Divalent free solution - 140, NaCl; 6, KCl; 5, D-glucose; 10, HEPES; 0.5, EGTA; (3) K^+ -based pipette solution - 138, KCl; 1, MgCl_2 ; 0.5, EGTA; 0.2, CaCl_2 ; 10, HEPES; pH set to 7.2 with KOH. (4) Cs^+ -based pipette solution - 138, CsCl; 1, MgCl_2 ; 0.2 CaCl_2 ; 10, HEPES; 0.5, EGTA; 2, ATP-2Na; 0.1, GTP-2Na; pH set to 7.2 with CsOH; (5) Nominal Ca^{2+} free solution was of Hanks' composition, only Ca^{2+} was omitted. Amphotericin B (600 mg/ml) was dissolved in the pipette solutions as the pore-forming agent.

Unless otherwise stated, stock solutions of drugs were initially prepared in DMSO and then diluted to the final concentration. The final bath concentration of DMSO was $\leq 0.01\%$. 4-aminopyridine (4-AP), amphotericin B, arachidonic acid, disodium 4,4'-diisothiocyanatostilbene-2,2'-disulfonate (DIDS), EGTA, 5,8,11,14-Eicosatetraynoic acid (ETYA) and penitrem A were purchased from Sigma (Poole, UK). Cyclopiazonic acid and nimodipine were from Alexis Biochemicals (Exeter, UK) and Fluo-4AM was from Molecular Probes Inc. (Eugene, Oregon, USA). Ryanodine was purchased from Ascent Scientific (Bristol, UK) and dissolved in absolute ethanol (bath vehicle concentration of 0.1%). All drugs were introduced abluminally via superfusion at 2-3 ml/min. Bath solutions were maintained at 37°C by passing the solution through a heat exchanger.²⁵

In vehicle control experiments, application of either DMSO or ethanol, at the maximal concentrations used in these studies, had no effect on arteriolar diameter ($0.0 \pm 1.1\%$ for DMSO at 0.01% v/v, $n=5$ and $0.6 \pm 0.3\%$ for ethanol at 0.1% v/v, $n=6$; $P=0.94$ and $P=0.06$ respectively, paired t-test, Table 1). A difference in the properties of Ca^{2+} sparks was observed in vehicle control experiments with DMSO (0.01% v/v). Specifically, it caused a small yet significant increase in the frequency of these events (from a control value of $0.040 \pm 0.020 \text{ cell}^{-1}\text{s}^{-1}$ to $0.058 \pm 0.011 \text{ cell}^{-1}\text{s}^{-1}$ after DMSO exposure, $n=78$, $P<0.01$, Mann Whitney U-test). Note, however, that the action of DMSO on Ca^{2+} spark frequency was opposite to the effect of AA (see results).

Statistical analysis

Data are presented as the mean \pm SEM. The statistical significance of differences in arteriole diameter under control and experimental conditions were determined using a paired t-test or repeated measures ANOVA followed by Bonferroni multiple comparison tests as appropriate. Paired data for the percentage of smooth muscle cells displaying Ca^{2+} sparks and/or Ca^{2+} oscillations before and during AA treatment were tested using the Wilcoxon signed-rank test. Spark or oscillation population data, consisting of all spatiotemporal measurements for individual Ca^{2+} events, were compared using the Mann Whitney U-test. Summary frequency data were generated by averaging the frequency seen in each cell, with inactive (quiescent) myocytes counted as zero. Differences in whole-membrane current densities and time constants of inactivation were tested with two-way ANOVA or paired t-test. In all comparisons of mean data, the 95% level was accepted as statistically significant. In all graphical representations of the data, statistical significance is indicated as follows: NS, $P>0.05$; *= $P<0.05$; **= $P<0.01$; ***= $P<0.001$.

RESULTS

AA-induced vasodilation and inhibition of Ca^{2+} sparks and oscillations

Because physiological concentrations of AA are frequently in the low-to-mid micromolar range (1-150 μM),²⁶ we chose to test AA at a concentration of 10 μM . Application of 10 μM AA (5 min) to isolated, pressurized rat retinal arterioles resulted in an increase in vessel diameter that averaged $9.2 \pm 1.3\%$ of the steady-state diameter under conditions of myogenic tone ($P < 0.001$, repeated measures ANOVA, Fig 1Ai-Ci and Table 1). This effect was irreversible within the time limit of the wash out period (5 min; $7.2 \pm 1.9\%$, $P < 0.001$ vs baseline). The non-metabolizable analogue of AA, ETYA (10 μM), also elicited dilation of myogenically active, pressurized rat retinal arterioles (diameter increase of $13.7 \pm 4.2\%$; $P < 0.01$; Fig 1Aii-Cii and Table 1), but unlike AA, its effects were rapidly reversible (5 min; $3.8 \pm 4.9\%$ of the initial steady-state diameter prior to ETYA addition; $P > 0.05$ vs baseline).

Ca^{2+} sparks are brief, highly localized subcellular Ca^{2+} release events. In retinal arteriolar myocytes, Ca^{2+} sparks can summate to generate more prolonged global Ca^{2+} oscillations, which in turn can lead to myocyte contraction and the generation of myogenic tone.^{22, 27} We therefore tested whether AA modulates sub-cellular Ca^{2+} signalling events in these vessels. Bright field (vessel diameter) and confocal (Ca^{2+} -signalling) images were recorded sequentially from myogenically active arterioles maintained at 70 mmHg and loaded with the fluorescent Ca^{2+} indicator dye, Fluo-4. The average AA-induced vasodilation in these vessels was $6.6 \pm 0.9\%$ ($P < 0.001$, paired t-test). This did not differ significantly from the vasodilatory response observed in unloaded vessels ($P = 0.17$; Mann Whitney U-test).

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Under control conditions, myogenically active arterioles exhibited spontaneous sub-cellular Ca^{2+} signalling activity, as previously described.²² AA-induced vasodilatory effects were associated with a pronounced inhibition of sub-cellular Ca^{2+} signals (Fig. 2). The percentage of smooth muscle cells displaying Ca^{2+} sparks decreased from $52.3 \pm 5.1\%$ under control conditions to $23.8 \pm 8.9\%$ following AA exposure ($P < 0.05$, Wilcoxon signed-rank test). With the exception of frequency, which decreased from $0.066 \pm 0.013 \text{ cell}^{-1}\text{s}^{-1}$ under control conditions to $0.019 \pm 0.005 \text{ cell}^{-1}\text{s}^{-1}$ after AA addition ($P < 0.001$, Mann Whitney U-test), other spatiotemporal features of Ca^{2+} sparks were unaffected (Table 2).

AA also altered the percentage of smooth muscle cells displaying Ca^{2+} oscillations, which decreased from $70.0 \pm 2.4\%$ under control conditions to $43.9 \pm 9.8\%$ after application of AA ($P < 0.05$, Wilcoxon signed-rank test). The average frequency of Ca^{2+} oscillations was also decreased, from a control value of $0.078 \pm 0.008 \text{ cell}^{-1}\text{s}^{-1}$ to $0.031 \pm 0.005 \text{ cell}^{-1}\text{s}^{-1}$ during AA exposure (Table 2; $P < 0.001$ Mann Whitney U-test). AA also reduced the amplitude and duration of these Ca^{2+} events. When averaged, the peak amplitude (F/F_0) was 2.17 ± 0.07 in control and 1.70 ± 0.07 following AA application ($P < 0.05$, Mann Whitney U-test), while the full duration at half maximum (FDHM) decreased from $866.6 \pm 0.03 \text{ ms}$ under control conditions to $770.6 \pm 0.05 \text{ ms}$ after AA addition ($P < 0.01$, Mann Whitney U-test).

Since AA blocked sub-cellular Ca^{2+} signals in pressurized rat retinal arterioles, we wanted to explore in more detail whether these effects were causally related to the dilatory response. As shown in Table 3, pharmacological interventions that are known to block Ca^{2+} sparks and Ca^{2+} oscillations in retinal arterioles²⁸ inhibited the AA-induced vasodilatory response. In general, these results could not be attributed to the vessels reaching their maximal vasodilatory

capacity prior to AA exposure since in the majority of vessels tested (>70%) a greater dilation could be evoked by applying nominal Ca^{2+} free solution than in the presence of the various Ca^{2+} signalling inhibitors (see, for example, Supplementary Fig 1).

AA-modulation of ion channels in arteriolar smooth muscle cells

Studies in other vascular beds have demonstrated that AA-induced vasodilation depends on modulation of K^+ channel function.^{16, 29, 30} We therefore decided to examine the effects of AA on the major K^+ currents (A-type K_v and BK currents) previously characterized in retinal arteriolar smooth muscle cells.^{23, 31, 32}

Following AA exposure (10 μM) the sustained component of the A-type K_v current was inhibited at potentials between +20 mV and +100 mV, whereas the maximal (peak) current was unchanged (Fig. 3A-B). Inspection of the kinetics of the A-type K_v current profile revealed a dramatic increase in the inactivation rate following treatment with AA. In the presence of AA, the mean time constant of fast inactivation was reduced from 37 ± 3 ms to 16 ± 7 ms ($P < 0.05$ paired t-test), while the mean time constant of slow inactivation decreased from 370 ± 64 ms to 77 ± 19 ms ($P < 0.001$, paired t-test; Fig. 3C). To establish the range of membrane potentials over which K_v channels are capable of mediating a sustained outward 'window current', we examined the voltage-dependence of activation and inactivation before and after AA exposure (Fig. 3D). We found that the voltage-dependence of activation was not altered in the presence of AA ($P = 0.52$, repeated measures two-way ANOVA). In contrast, the voltage dependence of inactivation was shifted negatively by AA. Under control conditions the A-type K_v current was

fully inactivated at -22.7 ± 2.4 mV, whereas following AA treatment full inactivation was observed at -35.7 ± 1.9 mV ($P < 0.01$, paired t-test).

Voltage-activated BK currents were investigated in retinal arteriolar myocytes by stepping the membrane potential from -80 mV to $+70$ mV in 10 mV increments. Using this protocol, changes in BK currents following the addition of $10 \mu\text{M}$ AA are illustrated in Fig. 4Ai. It is apparent that AA treatment substantially enhanced these currents, but only at membrane potentials positive to $+30$ mV (Fig. 4Aii). We also examined spontaneous BK currents at steady-state holding potentials between -60 mV and $+60$ mV.³² Similar to our findings for voltage-activated BK currents, spontaneous BK currents were significantly increased by $10 \mu\text{M}$ AA, but only at holding potentials positive to $+20$ mV (Fig 4Bi,ii).

To further examine the possible contribution of A-type K_v and BK currents in the AA-induced dilation of pressurised retinal arterioles, a set of experiments was carried out in the presence of the A-type K_v and BK channel blockers, 4-AP (10 mM) and Penitrem A (100 nM), respectively. Addition of the K^+ channel blockers to myogenically active retinal arterioles resulted in significant vasoconstriction ($6.4 \pm 1.3\%$ decrease in vessel diameter; $P < 0.01$, repeated measures ANOVA, Table 3). Subsequent addition of AA in the continued presence of the K^+ channel blockers evoked a robust vasodilatory response ($8.0 \pm 1.8\%$ increase in vessel diameter; $P < 0.01$, repeated measures ANOVA, Table 3) that did not differ significantly from that previously observed with AA alone ($p = 0.82$; Mann Whitney U-test).

Data from our own laboratory have shown that Ca^{2+} influx through L-type Ca^{2+} channels contributes to myogenic tone development and the generation of sub-cellular Ca^{2+} signalling activity in retinal arterioles.²⁷ It was therefore of interest to examine the effects of AA on these

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channels. As shown in Fig. 5, L-type Ca^{2+} currents were AA-sensitive. On average, the peak inward current during voltage steps from -80 to 0 mV decreased from -11.7 ± 3.5 pA/pF under control conditions to -2.4 ± 2.7 pA/pF after AA treatment ($P < 0.05$, paired t-test).

Discussion

AA-induced modulation of myogenic tone and subcellular Ca^{2+} signalling

The results presented here provide the first direct evidence for a role of AA in modulating retinal vascular tone. Our findings are consistent with previous reports showing that AA is capable of mediating vasodilation in coronary,³³ mesenteric,^{3, 34} femoral,³⁵ intrapulmonary³⁶ and placental artery.³⁷ They also demonstrate that AA is not only an important mediator of vasodilation in large arterial vessels, but also acts at the level of the microcirculation.

Confocal Ca^{2+} imaging in pressurised retinal arterioles demonstrated that sub-cellular Ca^{2+} signals in retinal arteriolar myocytes are inhibited by AA. To the best of our knowledge, this is the first direct evidence demonstrating that AA is capable of modulating localised Ca^{2+} signals in any tissue. Our data also strongly supports the idea that inhibition of Ca^{2+} spark and Ca^{2+} oscillation activity is the primary mechanism underlying the effects of AA on retinal arteriolar tone, since interventions that blocked Ca^{2+} sparks and Ca^{2+} oscillations prevented the vasodilatory effects of AA (although we cannot fully rule out the possibility that a few of the vessels included in this dataset had already reached their maximal vasodilatory capacity prior to AA addition). Whilst this study is the first to describe the effects of AA on sub-cellular Ca^{2+} signalling, previous studies have reported changes in Ca^{2+} sparks and global Ca^{2+} signalling activity in response to other polyunsaturated fatty acids (PUFAs). For instance, in cardiac myocytes, the omega-3 PUFA, eicosapentaenoic acid (EPA), has been found to reduce both the width and duration of Ca^{2+} sparks³⁸ and the amplitude and rate of propagation of spontaneous Ca^{2+} waves.³⁹ Taken together, these results and those of the present study suggest that the suppression of sub-cellular Ca^{2+} signalling activity may represent a common mechanism

underlying the physiological effects of PUFAs in both cardiac myocytes and vascular smooth muscle cells.

AA-induced modulation of K⁺ currents

From a physiological perspective, it is well established that the activation of K⁺ channels counteracts the development of myogenic tone by providing a hyperpolarising influence which reduces Ca²⁺ influx through voltage-dependent Ca²⁺ channels and thereby limits the degree of vasoconstriction.⁴⁰ K⁺ channel activation has been implicated in the dilator effects of AA in a number of vascular beds.^{13, 16} However, as described below, our electrophysiological data are inconsistent with the idea that K⁺ channels contribute to the vasodilator effects of AA in retinal arterioles, and notably, we observed no significant difference in the degree of AA-induced vasodilation in the absence and presence of K⁺ channel inhibitors.

We found that AA caused acceleration in the time course of inactivation and a negative shift in the voltage-dependence of inactivation of the A-type K_V current in retinal arteriolar myocytes, reducing the steady state K_V window current. A reduction in K_V window current would be expected to cause cell membrane potential depolarisation, increased Ca²⁺ influx through voltage-dependent Ca²⁺ channels and thus vessel constriction rather than dilation. Our observation of accelerated inactivation concurs with previous studies examining the effects AA on fast inactivating K_V currents in rat pituitary melanotrophs⁴¹ and rat hippocampal and pyramidal neurons.^{42, 43} Studies using heterologous expression systems suggest that, at a molecular level, AA increases the rate of inactivation of K_V channels by inducing conformational alterations in the selectivity filter.⁴⁴

We found that AA increased the amplitude of voltage-activated and spontaneous BK currents in retinal arteriolar myocytes. These findings are consistent with previous observations in gastric myocytes of guinea pig and rabbit pulmonary artery smooth muscle cells.^{45, 46} Recently, it has been reported that AA may act to potentiate BK currents through an indirect mechanism involving metabolites of the cyclooxygenase (COX) pathway.²⁹ Although we observed similar effects of AA on BK currents as those reported in other types of smooth muscle, it is important to note that the resting membrane potential of retinal arteriolar myocytes is far more negative (-40 mV)²³ than the threshold for AA-induced activation of BK currents in these cells (+40 mV; Fig 4B). Thus, it would appear that this mechanism is not likely to be relevant at physiological potentials and this most likely explains why inhibition of these channels failed to attenuate the vasodilatory actions of AA on myogenically active retinal arterioles.

AA effects on L-type Ca^{2+} currents

The results of present study show that AA inhibits L-type channel currents. Similar findings have been reported for smooth muscle cells from the vas deferens of the guinea-pig⁴⁷ and rabbit intestine⁴⁸ and in cardiac myocytes from frog ventricle.⁴⁹ There is no consensus on the mechanism responsible. Khurana and Bennett (1993) suggested that in ciliary ganglion cells leukotrienes synthesized from AA by lipoxygenase act as second messengers responsible for AA-induced block of voltage-dependent Ca^{2+} channels.⁵⁰ Other studies, meanwhile, have reported that the inhibition of voltage-gated Ca^{2+} channels by AA may, at least in part, be due to superoxide radicals derived from AA oxidation.⁴⁷ Regardless of the mode of action at the ion

channel level, our results are consistent with the idea that the effects of AA on sub-cellular Ca^{2+} signalling and hence vascular tone in retinal arterioles are most likely due to the inhibition of voltage-dependent Ca^{2+} entry. Indeed in previous work, we have shown that blockade of L-type Ca^{2+} channels suppresses Ca^{2+} sparks and oscillations in retinal arterioles, and causes vasodilation through a pathway associated with a reduction in the sarcoplasmic reticulum (SR) Ca^{2+} content.²⁷ It is worth stressing, however, that at this stage other possible contributory mechanisms cannot be fully discounted. For example, possible effects of AA on sarcoplasmic/endoplasmic reticulum Ca^{2+} -ATPase (SERCA) activity and store-operated Ca^{2+} entry could also contribute to the inhibition of sub-cellular Ca^{2+} signalling activity by modifying SR Ca^{2+} content. It is also possible that AA may act to block the function of ryanodine receptors on the SR, which are known to underlie Ca^{2+} spark and oscillation activity in retinal arteriolar myocytes.²⁷ Previous biophysical studies using lipid-bilayer systems, however, have suggested that AA is not a direct inhibitor of these channels.²⁰

Conclusions

In conclusion, our experiments provide evidence that AA contributes to the modulation of the contractile, Ca^{2+} signalling and cell membrane ion channel activity of retinal arteriolar myocytes. Our patch clamp data and pressure myography experiments suggest that AA actions on individual K^+ conductances cannot explain the vasodilatory effects of this lipid signalling molecule. The most likely mechanism underlying the actions of AA on retinal arteriolar tone is through a pathway involving the inhibition of Ca^{2+} sparks and Ca^{2+} oscillations secondary to blockade of L-type Ca^{2+} channels. A major question that remains is whether these effects are

exerted directly through AA or result from the generation of AA metabolites. Our studies using the non-metabolizable analogue of AA, ETYA, suggest that AA may be capable of directly causing retinal arteriolar vasodilation, although differences in the reversibility of these compounds indicates that a contribution by AA metabolites cannot be fully excluded. Elucidating a possible role for AA metabolites in the vasodilator effects of AA on retinal arterioles provides a clear direction for future research in this area.

TABLES

Table 1. Mean (\pm SEM) changes in internal diameter of pressurized retinal arterioles in response to AA, ETYA and vehicle controls.

Baseline diameter (μm)	Max diameter in presence of drug (μm)	Diameter following 5 min of wash out
28.3 \pm 1.5	AA (10 μM ; n=11) 30.8 \pm 1.5***	30.3 \pm 1.6***
25.5 \pm 2.5	ETYA (10 μM ; n=8) 28.5 \pm 2.4**	26.0 \pm 2.2 NS
27.7 \pm 3.0	DMSO (0.01% v/v; n=5) 27.7 \pm 3.0 NS	NA
27.4 \pm 1.4	Ethanol (0.1% v/v; n=6) 27.6 \pm 1.5 NS	NA

** P<0.01, *** P<0.001 versus 'Baseline'; t-test or repeated measures ANOVA.

Table 2. The percentage of myocytes exhibiting Ca^{2+} sparks and oscillations and the spatiotemporal features of these events in pressurized rat retinal arterioles under control conditions and following AA treatment.

	Ca^{2+} sparks		Ca^{2+} oscillations	
	Control	AA	Control	AA
Active cells (%)	52.3 \pm 5.1 (105)	23.8 \pm 8.9 [‡] (85)	70.0 \pm 2.4 (105)	43.9 \pm 9.8 [‡] (85)
Amplitude (F/F ₀)	1.53 \pm 0.06 (414)	1.55 \pm 0.11 (99)	2.17 \pm 0.07 (493)	1.70 \pm 0.07* (156)
FDHM (ms)	23.99 \pm 0.88 (190)	23.07 \pm 1.92 (41)	866.6 \pm 0.03 (493)	770.6 \pm 0.05** (493)
FWHM (μm)	0.46 \pm 0.01 (414)	0.41 \pm 0.01 (99)	NA	NA
Frequency (cell ⁻¹ sec ⁻¹)	0.066 \pm 0.013 (105)	0.019 \pm 0.005*** (85)	0.078 \pm 0.008 (105)	0.031 \pm 0.005*** (85)

FDHM: full duration at half maximum; FWHM: full width at half maximum. Summary data are means \pm SEM obtained from 11 vessels (10 animals) during pressure induced steady-state constriction (control) and 5 min after an application of AA (10 μ M). * = $P < 0.05$, ** = $P < 0.01$ and *** = $P < 0.001$ versus 'control' by Mann Whitney U-test; ‡ = $P < 0.05$ versus 'control' by Wilcoxon sign-rank test; Numbers in parentheses represent the number of Ca^{2+} events with the exception of frequency and the percentage of active myocytes where the number of cells is given.

Table 3. Mean (\pm SEM) changes in internal diameter of pressurized retinal arterioles in response to AA following pre-treatment with pharmacological inhibitors. Maximum diameter was recorded after development of myogenic tone ('Baseline') and following cumulative addition of pharmacological inhibitors and arachidonic acid (AA), as indicated.

Baseline diameter (μ m)	Max diameter in presence of inhibitor (μ m)	Max diameter in presence of inhibitor and AA (10 μ M) (μ m)
28.5 \pm 1.8	Ryanodine (100 μ M; n=9) 31.5 \pm 1.1*	Ryanodine + AA 32.4 \pm 1.1**
29.3 \pm 1.0	CPA (20 μ M; n=5) 31.6 \pm 1.0***	CPA + AA 31.6 \pm 0.9***
30.3 \pm 1.5	Nimodipine (1 μ M; n=6) 32.5 \pm 1.4**	Nimodipine + AA 33.3 \pm 1.6***
28.4 \pm 3.8	4-AP (10 mM) & Penitrem A (100 nM; n=5) 26.6 \pm 3.7**	4-AP + Penitrem A + AA 28.5 \pm 3.7

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ versus 'Baseline'; repeated measures ANOVA.

Ryanodine: blocks ryanodine receptors;²⁷ Cyclopiazonic Acid (CPA): inhibits SR Ca^{2+} uptake;⁵³ Nimodipine: inhibits L-type Ca^{2+} -channels²⁶, 4-AP and Penitrem A block A-type K_v^{23} and BK channels³², respectively.

Comment [T3]: This reference is needed, but we need to make sure it follows in the correct order in the main MS.

Table S1. Protocols used to isolate individual whole-membrane currents

Current of interest	Pipette solution	Protocol	Bath solution	Pharmacological inhibitors and their targets applied to isolate current of interest
A-type K_V	K^+ -based	Family of depolarizing steps from -80 to +100 mV	Standard Hanks' solution	100 nM Penitrem A (BK current) ³² 1 mM DIDS (Ca^{2+} -activated Cl^- current) ⁵¹ 1 μ M nimodipine (L-type Ca^{2+} current) ⁵²
BK	K^+ -based	Family of depolarizing steps from -80 to +70 mV	Standard Hanks' solution	10 mM 4-AP (K_V current) ²³ 1 mM DIDS (Ca^{2+} -activated Cl^- current) ⁵¹ 1 μ M nimodipine (L-type Ca^{2+} current) ⁵²
L-type Ca^{2+}	Cs^+ -based	Single voltage step from -80 mV to 0 mV, applied every 4 sec for ~1 min*	Divalent free solution to increase the current amplitude ⁵³	100 nM Penitrem A (BK current) ³² 10 mM 4-AP (K_V current) ²³ 1 mM DIDS (Ca^{2+} -activated Cl^- current) ⁵¹

* - the instability of the gigaseal in divalent free solution prevented a comprehensive characterisation of the effects of AA on the L-type Ca^{2+} current.

Comment [T4]: As this is supplementary information I think we need to have this as a separate file, with the references listed at the bottom i.e. references 51 and 52 shouldn't form part of the main MS.

Fig 1. Dilation of pressurized rat retinal arterioles in response to AA and its non-metabolizable analogue, Eicosatetraynoic acid (ETYA). (A) Time-course records showing changes in internal diameter of pressurized rat retinal arterioles during application of 10 μ M AA (i) and 10 μ M ETYA (ii). Bars represent drug application intervals. (B) Photomicrographs of the same arterioles at the time points indicated by arrows in Ai and ii. (C) Mean (\pm SEM) percentage changes in the diameter of arterioles superfused for 5 min with either 10 μ M AA (i) or ETYA (ii) and after 5 min of wash out. (Data was collected from a minimum of 8 vessels from 6 rats; ns = $P > 0.05$, ** = $P < 0.01$, *** = $P < 0.001$ relative to baseline; repeated measures ANOVA).

Fig 2. AA inhibits sub-cellular Ca^{2+} signals in pressurized rat retinal arterioles. Low (A) and high (B) temporal resolution confocal line scan images and time series plots showing changes in normalized fluorescence ($\Delta F/F_0$) for representative adjacent arteriolar myocytes (labelled from 'a' to 'h'). Paired images in A and B were recorded from two different regions of the same vessel under control conditions and 5 min after application of 10 μ M AA. The regions marked with the dashed boxes in A correspond to the panels shown below in B. Two types of sub-cellular Ca^{2+} signalling events can be observed: prolonged Ca^{2+} oscillations which are identifiable in both low and high temporal resolution images and brief Ca^{2+} sparks (indicated by asterisks in B) which can only be resolved in high temporal resolution images. Note a decrease in the frequency of both Ca^{2+} sparks and oscillations following AA treatment.

Fig 3. AA-induced modulation of A-type K_v current in retinal arteriolar myocytes. (A) A-type K_v currents evoked in response to depolarizing voltage steps (protocol shown in inset) under control conditions (i) and in the presence (ii) of 10 μ M AA (7 min). (B) Mean (\pm SEM) peak (i) and steady-state (ii) current densities recorded under control conditions (closed circles) and in the presence of 10 μ M AA (open circles; n=6). (Ci) A-type K_v currents evoked in response to a single voltage step from -80 mV to +40 mV in the absence and presence of AA (extracted from A). Currents were fitted with double exponential curves (solid lines superimposed on current traces) and inactivation time constants calculated. (Cii) Mean (\pm SEM) fast and slow time constants before and following AA exposure (n=8). Note a decrease in the time constants in the presence of AA indicating acceleration of the rate of K_v current inactivation. (Di) Representative current traces resulting from a double pulse protocol (shown in inset) applied to obtain the steady-state, voltage-dependent inactivation of the A-type K_v current in the presence and absence of AA. (Dii) The voltage dependence of activation (open circles, n=6) and inactivation (closed circles, n=7) were calculated as described in the methods and plotted against membrane potential. The lines are the fit of Boltzmann relationships to the data for control (solid line) and AA (dashed line) conditions. Under control conditions the activation and inactivation curves overlap substantially revealing a 'window current' (area under the two intersecting curves). Note that AA exposure resulted in a negative shift in the inactivation curve causing a reduction in the window current. Symbols *, **, *** denote $P < 0.05$, $P < 0.01$, and $P < 0.001$ significance level respectively.

Fig 4. AA-induced activation of the BK current in retinal arteriolar myocytes (Ai) BK currents evoked in response to depolarizing voltage steps (protocol shown in inset) under control conditions (upper panel) and following 5 min application of 10 μ M AA (lower panel). (Aii) Mean (\pm SEM) BK current densities (averaged across each depolarizing voltage step) under control conditions (closed circles) and in the presence of 10 μ M AA (open circles). Note an enhancement of BK current at membrane potentials positive to +30 mV (n=5; *P<0.05, ***P<0.001, two-way ANOVA). (Bi) Spontaneous BK currents recorded at various holding potentials under control conditions (left panel) and following 5 min application of 10 μ M AA (right panel). (Bii) Mean (\pm SEM) integrated spontaneous BK current densities under control conditions (closed circles) and in the presence of 10 μ M AA (open circles). Enhancement of spontaneous BK current activity was observed at holding potentials positive to +20 mV (n=4; ***P<0.001, two-way ANOVA).

Fig 5. AA-induced inhibition of the L-type Ca^{2+} current in retinal arteriolar myocytes. (A) L-type Ca^{2+} currents under control conditions and following ~1 min exposure to 10 μ M AA evoked by depolarizing steps from -80 mV to 0 mV (inset). (B) Mean (\pm SEM) peak L-type Ca^{2+} current densities for the experimental conditions shown in A (n=6; *P<0.05 paired t-test).

Fig. S1 Vasodilatory reserve of a retinal arteriole after treatment with ryanodine.

Original tracing showing myogenic tone development following an elevation in intraluminal pressure (arrows) and vasodilations in response to consecutive treatments with 100 μ M

ryanodine and nominal Ca^{2+} free solution. Note that the vasodilation in the presence of Ca^{2+} free solution exceeds the response observed with ryanodine.

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